Korean Hemorrhagic Fever: Propagation of the Etiologic Agent in a Cell Line of Human Origin

Abstract. *The etiologic agent of Korean hemorrhagic fever has been propagated in a human cultured cell line derived from a carcinoma of the lung. The cells, described as type II, alveolar epithelial, support replication of the agent and successive passages. Antigen of the Korean hemorrhagic fever agent is readily detected in infected cells by means of direct or indirect fluorescent antibody techniques. Previous attempts to propagate this agent in vitro had been unsuccessful.*

Korean hemorrhagic fever (KHF), which is presumed to be of viral origin, is one member of a group of similar hemorrhagic fevers with renal syndrome that occur throughout large portions of the world from Japan in the East, throughout Soviet Russia, to Sweden in the West (*1–4*).

H. W. Lee and co-workers reported recently the isolation of the KHF etiologic agent from a rodent, *Apodemus agrarius coreae* (*1*). Specific antigen for KHF was detected in various tissues of this rodent, by using an indirect fluorescent antibody (IFA) technique (*5*) and samples of serum from human patients recovering from KHF. Isolation of the KHF agent had been attempted by several groups of investigators since 1952 (*1, 2, 6–8*). Attempts to propagate the agent in cell culture have also been made, without success (*1, 3, 8, 9*). However, we now report the successful propagation of the etiologic agent of KHF in an in vitro substrate, a human cell line. This line, designated A-549 and described as type II, alveolar epithelial cells, was derived from a carcinoma of the lung (*10, 11*).

The starting infectious material for this study was a pooled suspension (10 percent) of lung tissue from several *A. a. coreae* killed 21 days after they were inoculated with pooled tissue from several *Apodemus* infected with fourth-passage KHF strain 76-118 (*1*). A 10^{-1} dilution of this material in medium E-199 containing 10 percent fetal calf serum (FCS) was inoculated onto monolayers of various primary and continuous avian and mammalian cells prepared on 12-mm glass cover slips in 24-well plates (*12*). The cover slip monolayers were maintained with medium E-199 with 5 percent FCS which was changed at 3- to 5-day intervals as required. Inoculated cultures were examined daily for cytopathogenic effect (CPE). On day 8 after inoculation and at 2-day intervals thereafter through day 24, two to three cover slips were removed, fixed in 100 percent cold acetone, and examined by the IFA technique for the presence of KHF-related antigen (*1*). In addition, occasional cover slips were stained at various intervals with acridine orange or May-Grünwald stain and examined for the presence of viral inclusions. The different cell cultures used in this and earlier studies are shown in Table 1.

The only cultured cells in which we could reproducibly demonstrate specific fluorescence or other evidence of infection were the A-549 cells. These cells were first recognized as positive on day 12 after inoculation, with the appearance of a single fluorescent focus of less than a dozen cells. Fluorescence appeared as discrete pinpoint granules distributed throughout the cytoplasm. Fluorescent foci increased in number and brightness on companion cover slips throughout the remaining observation period. Subsequent passages of this agent have led to 100 percent infection of the cells with a reduction in time of the first appearance of specific fluorescence to as little as 3 days after inoculation by the sixth passage in A-549 cells. At no time during the initial or subsequent passages in A-549 cells was CPE detected or were viral inclusions found by staining with acridine orange or the May-Grünwald technique.

Numerous attempts to induce plaque formation on A-549 cells have not been rewarding. Small indistinct plaques do form under agarose overlay medium, however, their occurrence is irregular and depends on conditions that stress the cells, and the plaques have not been useful for quantitative assay. Infected A-549 cells undergo cell division in an apparently normal manner and without loss of KHF-related specific fluorescence. Infected cells remain IFA-positive for at least 100 days, the longest period of observation to date. Maximum yield of cell culture-adapted agent is obtained at 36° to 37°C with input multiplicities (infectious units per cell) that ensure infection of all cells. Peak yields that average 10 to 15 infectious units per cell are obtained 33 to 42 hours after infection with an input multiplicity of three to five infectious units per cell. Continuous harvests of spent cell culture medium have been made at 24-hour intervals after this peak through the seventh week after infection, and yields average three infectious units per cell per harvest. Input multiplicities of less than one infectious unit per cell result in sharply lower yields during the acute phase of the growth cycle; however, yields nearly comparable to those at high input multiplicity occur once persistent infection is established.

We used serologic tests to establish that the agent isolated and propagated in A-549 cells was KHF-related. In one experiment we used serum samples from 32 humans, including 13 normal (non-KHF) subjects and 19 subjects in the acute or convalescent stage of KHF. We tested these samples by the IFA technique against monolayers of infected A-549 cells. The serums from 16 of the subjects, including nine who were normal or in the acute phase of KHF and from seven convalescents, were also tested simultaneously against frozen sections of lung tissue from KHF agent-infected *Apodemus*. These 16 serum samples had identical or very similar IFA titers in the two test systems. Six of the serum samples were paired specimens (obtained at the acute and convalescent stages) from three KHF patients; all three pairs showed significant (≥ fourfold) increases in IFA titer when tested with either substrate. The 16 remaining serums, which were lyophilized samples collected from well-documented KHF convalescent patients who were studied in Korea from 1966 to 1968 (*8*) also showed positive IFA titers with the infected A-549 cells.

In a second experiment, a human KHF convalescent serum conjugated with fluorescein isothiocyanate (prepared and lyophilized at the U.S. Army 406th Medical Laboratory-Japan in 1968) produced a titer of 1:160 in the direct fluorescent antibody test with infected A-549 cells and 1:160 when tested with frozen sections of infected *Apo-*

Table 1. The cultured cells used in this study (*13*) to isolate the etiologic agent of KHF.

Continuous cell lines
Human lung, alveolar epithelium (A-549)
Human adrenal cortex (SW-13)
African green monkey kidney (Vero)
Rhesus monkey kidney (LLC-MK_2)
African green monkey kidney (BSC-1)
Baby hamster kidney (BHK-21)
Rabbit vena cava, endothelium (REVC)
Dog kidney (Madin Darby)
Diploid cell lines
Human embryonic lung (WI-38)
Human embryonic lung (MRC-5)
Fetal rhesus lung (DBS-103)
Primary cell cultures
Human embryonic kidney
Human peripheral leukocytes
Microtus montanus whole embryo (vole)
Duck embryo fibroblasts
Calomys callosus kidney, lung, bladder
C. callosus alveolar and peritoneal macrophages
C. callosus peripheral leukocytes

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Table 2. Antigenic identity of the cell culture isolate to KHF agent passaged in *A. agrarius coreae*. The animals were inoculated intramuscularly with either a 15 percent suspension of lung tissue from the third passage of the KHF agent (Lee strain) in *Apodemus* or with A-549 cell culture fluids of the third or tenth cell culture passage with KHF agent, strain 76-118. The animals were bled on various days after inoculation. Serum antibody titers to the KHF agent were determined by the indirect fluorescent antibody (IFA) technique against frozen sections of infected *Apodemus* lung tissue or monolayers of infected A-549 cells. The IFA test procedure is described in (*1*).

Animal	Inoculum	Time after inoculation (days)	IFA titer by test system	
			A. a. coreae	A-549 cell
Rabbit	*A. a. coreae* lung P-3	0	< 16	< 16
		14	32,768	32,768
		45	4,096	4,096
Rabbit	A-549 cells P-10	0	≤ 16	≤ 16
		26	8,192	4,096
Owl monkey	*A. a. coreae* lung P-3	0	< 16	< 16
		35	2,048	2,048
Baboon	A-549 cells P-3	0	16	16
		35	512	512

demus lung. In a third experiment, several species of laboratory rodents or nonhuman primates were inoculated with either infected *Apodemus* lung suspensions or spent, cell culture media from the third or tenth passage A-549 cell culture agent. Serum samples obtained before or after inoculation were then tested in the IFA test against KHF-infected *Apodemus* lung sections or KHF-infected A-549 cells. All animals showed significant (≥ fourfold) increases in IFA titer by both test systems. Representative results with serum samples from two New Zealand White rabbits, a baboon (*Papio anubis*), and an owl monkey (*Aotus trivirgatus*) are presented in Table 2.

We conclude that the etiologic agent of KHF propagates and produces IFA detectable antigen in the A-549 human alveolar epithelial cell line. Reisolation of this agent from the fifth passage *Apodemus* (KHF strain 76-118) has been accomplished on three occasions. Characterization of the agent has not progressed as rapidly as might be hoped because of the low yield per cell, the difficulty we have experienced in separating the agent from cell debris, and the less than satisfactory assay system. However, our present results do indicate that the infectious unit is lipid solvent-sensitive and acid labile (pH ≤ 5), and that a very small proportion (≤ 10^{-4}) of the agent population will pass a membrane filter of 100-nm pore size (*1*). Curves showing growth of the agent after inoculation of A-549 cells with large numbers of infectious units reveal an eclipse phase of 6 to 8 hours in which less than 10^{-4} of the inoculum is recoverable, an observation that lends additional support to the long-standing hypothesis (*3*) that the etiologic agent of KHF is a virus.

Attempts to isolate the agent from humans and suspected rodent reservoirs in various geographic regions of the world have revealed that A-549 cells are not uniformly susceptible to all strains of the agent or related agents. On the Korean peninsula, for example, native *A. a. coreae* remains the host of choice for initial isolation of the agent. However, replication of KHF strain 76-118 in A-549 cells makes it possible to study the KHF agent in vitro and provides a means of diagnosing hemorrhagic fevers with renal syndrome in laboratories outside Korea. Although *A. a. coreae* is an adequate host for these purposes, the rodent has not been colonized and must be captured and returned to the laboratory from KHF-free areas of the Korean peninsula.

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5. The indirect fluorescent antibody test used in this study and all reagents, including the human reference positive control KHF antiserum and positive control rodent lung tissue, were described in Lee *et al.* (*1*). Samples of negative control serum were drawn from individuals who had never entered HFRS endemic areas [H. W. Lee, in *Ebola Virus Hemorrhagic Fever*, S. R. Pattyn, Ed. (Elsevier/North-Holland, Amsterdam, 1978), pp. 331–343].
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12. Tissue culture cluster-24, No. 3524, COSTAR, Cambridge, Mass.
13. Previous investigators have used other continuous and primary cell cultures, including dog embryo (R-1247), porcine kidney (PS), mink lung (MV1Lu), Chinese hamster lung (Dede), primary human embryonic lung, and primary rat liver (*1*); HEp-2, HeLa, FL, Detroit, six lines of human origin, and primary Korean hamster heart and kidney (*8*); rhesus monkey kidney (MA-104), newborn rabbit kidney (MA-111), primary hamster kidney (*5*), and rhesus monkey kidney (*1*, *5*, *8*).
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